Phosphoproteomics-based characterization of Prostaglandin E_2 signaling in T cells

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Abstract: Prostaglandin E_2 (PGE_2) is a key lipid mediator in health and disease, and serves as a crucial link between the immune response and cancer. With the advent of cancer therapies targeting PGE_2 signaling pathways at different levels, there has been increased interest in mapping and understanding the complex and interconnected signaling pathways arising from the four distinct PGE_2 receptors. Here, we review phosphoproteomics studies that have investigated different aspects of PGE_2 signaling in T cells. These studies have elucidated PGE_2's regulatory effect on T Cell Receptor signaling and T cell function, the key role of protein kinase A in many PGE_2 signaling pathways, the temporal regulation of PGE_2 signaling, differences in PGE_2 signaling between different T cell subtypes and finally, the crosstalk between PGE_2 signaling pathways elicited by the four distinct PGE2 receptors present in T cells.

Significance statement: Through the reviewed studies, we now have a much better understanding of PGE_2's signaling mechanisms and functional roles in T cells, as well as a solid platform for targeted and functional studies of specific PGE_2-triggered pathways in T cells.

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Introduction - Prostaglandin E₂ in T cell signaling and function[1]

The lipid mediator Prostaglandin E$_2$ (PGE$_2$) is the most abundant prostanoid in the human body and regulates key processes in normal physiology and disease, including in cancer (O'Callaghan and Houston, 2015) and in inflammatory conditions (Brudvik and Tasken, 2012). In particular, PGE$_2$ has received attention for the dual role it plays in the immune system as both a driver of acute inflammation and as an immunosuppressive mediator that contributes in the resolution phase of inflammation. PGE$_2$ thus constitutes an important link between the inflammatory response and cancer (Nakanishi and Rosenberg, 2013). PGE$_2$ is elevated in several different cancer types, including colon, lung and breast cancer, and is often associated with a poor prognosis (Wang and DuBois, 2013).

In the tumor microenvironment, PGE$_2$ is produced by tumor cells, monocytes and induced Tregs (iTregs) (Mahic et al., 2006; Scott et al., 2013) through an enzymatic cascade involving cyclooxygenase (COX) activity and Prostaglandin E synthase (PGES) (Tong et al., 2018). In this cancer setting, PGE$_2$ has established roles in promoting cancer cell proliferation, survival, migration and invasion well as in angiogenesis (Lone and Tasken, 2013). Aside from direct effects on tumor cells, PGE$_2$ acts on a number of the other cell types present in the tumor microenvironment, ultimately contributing to the formation of an immunosuppressive tumor microenvironment (Wang and DuBois, 2013). For instance, PGE$_2$ inhibits natural killer (NK) cell and dendritic cell (DC) function (De Keijzer et al., 2013) and promotes a shift from anti-tumor M1 to tumor-promoting M2-type macrophages (De Keijzer et al., 2013; Wang and DuBois, 2013; 2018). In T cells, PGE$_2$ has a number of distinct effects on apoptosis, activation-induced cell death, differentiation, and T cell function, including T Cell Receptor

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[1] Glossary, Key Immunology Terms. **TCR – T Cell Receptor**. A protein complex found on T cells that recognizes antigens presented by other cells and initiates an intracellular T cell signaling pathway in response, leading to T cell activation. **PGE$_2$ – Prostaglandin E$_2$**. A key lipid mediator, with roles in the immune system and in cancer. **EP$_{1-4}$** – The four GPCRs through which PGE$_2$ can signal. **T cell** – An important type of white blood cell. Part of the adaptive immune response. Roles in cancer, infection and more. **CD4 cell** – A T cell expressing the CD4 coreceptor. Also known as a T helper cell. Recognizes antigens presented on antigen-presenting cells, releases cytokines in response, thus helping shape the adaptive immune response. **CD8 cell** – A T cell expressing the CD8 coreceptor. Also known as a cytotoxic T cell. Recognizes antigens presented on other cells, and can directly kill the target cell. **Tregs – Regulatory T cell**. A T cell type that suppresses immune responses. **Effector/Memory T cells** – T cells that have encountered antigen. Express CD45RO. **Naïve T cells** – T cells that have not encountered antigen. Express CD45RA.

(TCR) signaling, proliferation, cytotoxicity and cytokine production (Lone and Tasken, 2013; Sreeramkumar et al., 2012).

PGE$_2$ mediates these effects on T cell function through four distinct G protein-coupled receptors (GPCRs), termed EP$_{1-4}$, all of which are present on T cells. The EP receptors initiate distinct and shared downstream pathways (Lone et al., 2021; O'Callaghan and Houston, 2015; Sreeramkumar et al., 2012; Woodward et al., 2011). The EP$_1$ receptor signals mainly through Gα_q, which activates PLC, Ca^{2+} and PKC signaling. The EP$_2$ and EP$_4$ receptors both couple to the stimulatory G protein Gα_s, which signals through cAMP elevation and PKA activation. In addition, EP$_4$ can also couple to the inhibitory G protein, Gα_i, and thus activate additional signaling pathways (Fujino and Regan, 2006; Yokoyama et al., 2013). EP$_3$ has several different isoforms that can couple to various G proteins, but the main signaling pathway is thought to occur through Gα_i. In addition to the canonical Gα signaling pathways, the EP receptors also signal through G$\beta\gamma$ subunits, in particular in conjunction with Gα_i signaling (Fujino and Regan, 2006; Fujino et al., 2002; Yokoyama et al., 2013). Further, G protein-independent signaling pathways, such as through beta-arrestin, have also been shown to occur downstream of some of the EP receptors (Buchanan et al., 2006; Chun et al., 2009; DeWire et al., 2007; Kim et al., 2010; Luttrell and Miller, 2013; Tan et al., 2017).

There has been significant interest in targeting some of these PGE$_2$ signaling pathways in cancer. For instance, the use of COX1/2 inhibitors, which block the rate-limiting step in PGE$_2$ biosynthesis, reduces colorectal cancer incidence (Rothwell et al., 2010) and improves survival if given after the initial diagnosis (Bains et al., 2016). PGE$_2$ receptors EP$_1$, EP$_2$ and EP$_4$ have also been targeted with antagonists, and there is significant interest in combining EP$_4$ antagonists with immunotherapy (Table 1). Previously, synergy between immunotherapy and other ways of targeting PGE$_2$ has been demonstrated. For instance, COX inhibitors enhance the effect of immune checkpoint blockade (Zelenay et al., 2015) and a peptide that blocks an inhibitory PGE$_2$ signaling pathway augments the antitumor efficacy of CAR T cells (Newick et al., 2016). Given the interest in targeting PGE$_2$ signaling pathways and their multifaceted roles in health and disease, it is important to have a thorough understanding of pathways, networks and functions regulated by PGE$_2$ in different cell types in health and disease.   Much of what is known about PGE$_2$ signaling has been discovered through classical biochemical signaling studies, which have identified and characterized specific PGE$_2$ signaling pathways and functional output in many different cell types, including in T cells (Brudvik et al., 2012; Brudvik and Tasken, 2012; Carlson et al., 2006; Lone and Tasken, 2013; Mahic et al., 2006; Mosenden et al., 2011; Ruppelt et al., 2007; Stokka et al., 2009; Vang et al., 2001). In recent years, however, large-scale proteomics studies have contributed to a more global view of PGE$_2$ signaling networks in different cell types, and this review article will focus on such studies in T cells.

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Phosphoproteomics and its application to signaling studies

Post-translational modifications (PTMs) are covalent changes to proteins after translation, and include a variety of modifications including, among others, ubiquitination, acetylation, methylation, proteolysis and phosphorylation (Walsh et al., 2005). These PTMs constitute an important mechanism for regulating protein localization, stability, protein-protein interactions, function and activity (Mnatsakanyan et al., 2018).

The best characterized PTM is protein phosphorylation, which is a rapid and reversible modification where phosphate groups are added to specific amino acid residues. Serine, threonine or tyrosine residues can be phosphorylated and the extent of phosphorylation is tightly regulated by kinases and phosphatases, which add and remove, respectively, phosphate groups at specific sites on proteins. As much as 30% of cellular proteins are thought to be phosphorylated (Cohen, 2000), and with more than 500 kinases and 100 phosphatases, approximately 3% of the human proteome is thought to be dedicated to the regulation of phosphorylation (Alonso et al., 2004; Manning et al., 2002). Phosphorylation events can change the conformation or binding properties of a protein, to yield changes in enzymatic activity, subcellular localization or stability (Alvarez-Salamero et al., 2017; von Stechow et al., 2015). Phosphorylation plays an important role in signal transduction, which in turn regulates key cellular processes such as cell division, proliferation, migration, differentiation, and survival (Alvarez-Salamero et al., 2017). Dysregulation of phosphorylation is frequently observed in cancer, metabolic disorders and immune conditions (Cohen, 2014; Lahiry et al., 2010; Needham et al., 2019).

Until recently, phosphorylation events were largely studied individually, using biochemical methods. The advent of mass spectrometry (MS) methods has enabled the assessment of phosphorylation levels on a more global level in a cell. These techniques began picking up speed a little more than a decade ago, and since then, there have been significant advances in techniques and instrumentation which allow for the characterization of ever-increasing numbers of phosphosites in a given experiment (Grimsrud et al., 2010; Lemeer and Heck, 2009; Macek et al., 2009). One of the main technical advances is in the instrumentation, where the ever-increasing acquisition speed and sensitivity of mass spectrometers have contributed to increased depth and throughput of proteomics studies (Riley and Coon, 2016; von Stechow et al., 2015). Further, sample fractionation methods have contributed towards deeper coverage of the phosphoproteome by reducing the complexity of the MS samples, as have developments in phosphoprotein enrichment strategies which isolate the phosphoproteome prior to MS analysis, thus compensating for the relatively low abundance of phosphopeptides compared to unmodified peptides (Riley and Coon, 2016). Data acquisition

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methods such as data-independent-analysis are also beginning to remedy the challenge of the wide dynamic range of the phosphoproteome, caused by the sub-stoichiometric nature of phosphorylation (Chapman et al., 2014; Needham et al., 2019; Riley and Coon, 2016). Thanks to these advances, current studies routinely characterize tens of thousands of phosphosites in a single experiment (Riley and Coon, 2016).

In immunology, proteomics is coming to play an important role, for instance in the study of protein expression levels, subcellular localization, secretion, interaction and for studying post-translational modifications (Nyman et al., 2017). In particular, phosphoproteomics studies are beginning to provide a more global pictures of the phosphorylation landscapes in immune cells. In T cells, phosphoproteomics has been used to study a wide variety of signaling processes, including TCR, IL2 and chemokine signaling, as well as signaling in various pathophysiological conditions, such as HIV infection and various T lymphocyte-mediated diseases (Alvarez-Salamero et al., 2017; Helou and Salomon, 2015). In the future, it might also be interesting to use phosphoproteomics to assess the effect of promising new cancer therapies such as immune checkpoint inhibitors on protein phosphorylation patterns in T cells. A handful of studies have also begun to use phosphoproteomics to shed light on PGE_2 signaling in T cells and this will be the focus of this review.

Phosphoproteomics studies of PGE_2 signaling in T cells

In the past decade, mass spectrometry-based phosphoproteomics studies have contributed to a much broader and more detailed overview of PGE_2 signaling in T cells (Table 2). A common factor in these studies has been an interest in characterizing entire signaling networks downstream of stimulation with PGE_2 and how these might affect T cell function. While some studies have focused specifically on PKA signaling networks, which are known to be major contributors to PGE_2 signaling output (Giansanti et al., 2013), other studies have looked more broadly at all PGE_2-initiated signaling events. Below, we will review common and specific themes and insights from these studies.

PKA Signaling and Interaction with TCR Signaling

One of the main biological insights resulting from these studies is that PKA phosphorylation plays a central role in the signaling downstream of PGE_2. Previous biochemical and cellular studies had implicated PKA in specific signaling pathways downstream of PGE_2, and in particular had identified a PKA-mediated inhibitory pathway that proceeds through EP_2/EP_4, cAMP/PKA, non-receptor tyrosine

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kinases Csk and Lck and ultimately leads to inhibition of TCR signaling, which is crucial for T cell function (Vang et al., 2001). A combined phosphoproteomics and phosphoflow cytometry study on PGE_2 signaling in primary lymphocytes demonstrated the significance of this pathway in T cells. First, the phosphoproteomics study revealed that PGE_2 regulated a number of phosphosites on proteins found downstream of TCR, such as CARD11, PLCG1, WIPF1, GRAP2, NFATC2, FYB1 and NCK1. (Figure 1, Table 3). Phosphoflow cytometry, which uses fluorophore-labeled phosphospecific antibodies to assess intracellular phosphorylation levels in a high-throughput manner by flow cytometry, was then used to follow up on these findings. The phosphoflow cytometry study demonstrated that the level of basal signaling through the inhibitory pathway in specific cell types sets the threshold for TCR signaling in primary T cells (Oberprieler et al., 2010), with CD8CD45RO cells exhibiting particularly high constitutive signaling through the inhibitory pathway and consequently low TCR signaling. Exogenously added PGE_2 had a similar dampening effect on TCR signaling, regardless of the basal signaling through PKA. To follow up on these insights, a further, more focused study was undertaken that demonstrated that this mechanism is also active in colorectal cancer patients, where high levels of circulating PGE_2 limits TCR and IL2 signaling in peripheral T cells (Moltu et al., 2017). This example highlights how phosphoproteomics and phosphoflow cytometry can complement more detailed molecular studies, and together characterize both specific pathways as well as the functional impact of these, as for the interaction between the PGE_2 and TCR signaling pathways. It also demonstrates that the combination of phosphoproteomics with phosphoflow cytometry can be a powerful way of achieving both a global overview as well as detailed knowledge about the kinetics, dose-dependency and cell-type specificity of specific regulated events (Oberprieler and Tasken, 2011).

Further evidence for the general importance of PKA in PGE_2 signaling was provided by a targeted phosphoproteomics study, which delved deeper into the importance of PGE_2 as a regulator of PKA signaling (Giansanti et al., 2013). In this study, an antibody specific for the PKA phosphorylation motif ([R/K][R/K/X]X[pS/pT]) was used to enrich phosphorylated PKA substrates triggered by PGE_2, thus zooming in on this particular signaling pathway, as opposed to the more common nonselective, shotgun phosphoproteomics approaches. Because of the enrichment, relatively few sites were identified (655 peptides, of which 642 had the PKA motif). Interestingly, the overlap with a large-scale Jurkat phosphoproteomics dataset was only 0.2%, illustrating the power of targeted approaches in complementing untargeted studies by generating unique information. Stimulation conditions were different in the two studies, so complete overlap would not be expected, but this nonetheless illustrates the fact that in untargeted studies, less abundant sites may be selected against due to data-dependent acquisition, and a targeted study may thus provide unique access to sites that otherwise would not be detected. These predominantly new phosphosites provide a useful

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resource of potential PKA substrates in T cells, and illustrate how a targeted approach can be useful to gain information on targets of a particular kinase. In addition, based on the basic substrate motif used for enrichment of potential substrates, additional information was gained about PKA motifs in real PKA substrates. In particular, the authors found that small residues are common in the P-1 position and hydrophobic residues are common in P+1. In addition, they identified some unconventional PKA motifs where histidine substitutes as a basic residue at position P-3. In other words, this targeted approach was used to both obtain a list of novel potential PKA substrates and gain new information about possible PKA substrate motifs. All in all, the study underlined the importance of PKA in PGE_2 signaling and mapped out the PKA-regulated substrates that are PGE_2-regulated. Further, it showed the potential of zooming in on particular pathways using enrichment-based mass spectrometry methods, which often provide high selectivity and orthogonality relative to shotgun approaches.

A general problem in detecting substrates of AGC kinases, such as PKA, by phosphoproteomics, is the frequent use of trypsin to digest proteins into peptides detectable by mass spectrometry. Of the studies discussed here, both Oberprieler et al., De Graaf et al., and Lone et al. used trypsin either alone or together with Lys-C. Trypsin cleaves after R/K, and will therefore cleave close to many PKA-targeted phosphosites conforming to the motif RRXS/T. However, PKA substrates still appear to be detected quite efficiently by MS in the trypsin-based studies. For instance, in Lone et al., the sequence motifs of regulated phosphosites show a clear enrichment for R/K in positions -2/-3 for most stimulation condition. In addition, several classic PKA substrates, such as BAD pS118 and CAD pS1406, were detected in the study. The other trypsin-based studies also detected many known PKA substrates.

Phosphodiesterases (PDEs) are enzymes responsible for breaking down cAMP, and are known to be phosphorylated and regulated by PKA, which was also observed in several of the studies described here. For instance PDE3B S442, which is situated in a typical PKA recognition motif, was regulated by EP1 and EP2 agonists in CD8 T cells in one study (Lone et al., 2021). A recent study on PDEs further highlighted the complexity of PGE_2 cAMP-PKA mediated signaling, the interaction with different PDEs, and the multiple possible roles of cAMP signaling upon PGE_2 stimulation (Beltejar et al., 2017). Here, the authors demonstrated that inhibition of different groups of PDEs in the presence of PGE_2 resulted in the upregulation of distinct phosphoproteomes and distinct functional compartments, underlining the downstream complexity of PGE_2-mediated cAMP-PKA signaling in T cells.

A more recent study has also underlined the significance of the PKA node in PGE_2 signaling (Lone et al., 2021) and further delineated its importance in signaling deriving from the four different EP receptors. In particular, the study demonstrated the predominance of PKA signaling in both EP_2 and EP_4 receptor signaling using phosphoflow cytometry, with EP_2 demonstrating more intense and longer duration of PKA-mediated signaling. The intensity difference is likely due to EP_4's secondary coupling to $G\alpha_i$, while the difference in signaling duration may be due to EP_4's higher susceptibility to internalization and desensitization than EP_2, caused by its longer intracellular C-terminal (Bastepe and Ashby, 1999; Desai et al., 2000; Nishigaki et al., 1996). It appears that EP_2 is less able to bind beta-arrestins than EP_4, but may bind certain arrestins to some extent (Chun et al., 2009; Penn et al., 2001), either as part of the desensitization process or in the context of G-protein independent signaling, which we incidentally observed a significant amount of in our recent study (Lone et al., 2021). In addition, the phosphoflow part of that study showed some evidence of phosphosites responding in opposite directions upon EP_3 or $EP_{2/4}$ stimulation. This would be expected, since EP_3 is thought to mainly couple to $G\alpha_i$, reducing intracellular cAMP and PKA signaling, and $EP_{2/4}$ are thought to primarily couple to $G\alpha_s$, increasing intracellular cAMP and PKA signaling. Some evidence of potentially opposing effects of EP_3 and $EP_{2/4}$ was also observed in the mass spectrometry part of the study, where EP_2 stimulation resulted in more regulated sites than PGE_2 stimulation, indicating that PGE_2 signaling, which would be expected to occur through all four EP receptors, is not simply additive. In particular, EP_3 signaling may dampen the effects of $EP_{2/4}$ signaling when all receptors are triggered simultaneously. It is interesting that nature has provided such a complex system of four distinct receptors all responding to the same stimulus, and in particular two receptors, EP2 and EP4, which both signal primarily through $G\alpha_s$. "Presumably, the crosstalk between the receptors, the difference in signaling intensity and duration (in particular between EP2 and EP4), and differences in coupling capabilities between receptors, as well as differences in relative receptor expression between cell types, allows this family of four receptors to provide a more fine-tuned response to PGE_2 stimulus than any one receptor could alone.

Further underlining the importance of PKA in PGE_2 signaling networks, PKA also assumes a key position in the modeled networks deriving from this phosphoproteomics study, both for PGE_2 stimulation of all receptors as well as for specific stimulation of each EP receptor.

Non-PKA Signaling Nodes and Pathways

Undoubtedly, PKA is a major mediator of PGE_2 signaling, but phosphoproteomics studies have also showcased other important kinase nodes and signaling pathways regulated by PGE_2. In particular, a

combined phosphoproteomics and phosphoflow cytometry study (Oberprieler et al., 2010) implicated kinases such as CamKII and Akt as possible weaker signaling nodes, largely based on kinase predictions. A further study that looked individually at signaling through the four EP receptors (Lone et al., 2021) also suggested PKC, CDKs, CK2, MAPKs, PI3K and Src as nodes involved in PGE$_2$ signaling, and used kinase inhibitors to confirm these. Interestingly, it appeared that the relative contributions of these kinases varied somewhat between cell types and naturally also between the EP receptors. Two further phosphoproteomics studies (de Graaf et al., 2014; Giansanti et al., 2013) supported the implication of the PI3K/Akt signaling pathway in PGE$_2$ signaling, and also identified a few other PGE$_2$-regulated kinases, including ROCK2 and MAPK1. Identifying these kinase nodes provides useful starting points for more targeted studies of PGE$_2$ signaling through its different receptors in different T cell types.

Signaling Networks Regulated by PGE$_2$

One of the challenges associated with large phosphoproteomics datasets is going beyond regulated nodes and pathways to visualize the complete and often complex signaling networks arising from a given stimulation (Needham et al., 2019). A major obstacle in this respect is the limited and somewhat skewed knowledge of kinase-substrate interactions, highlighted in a recent review (Needham et al., 2019). Here, the authors describe how the regulating kinase has only been identified for 5% of the phosphoproteome, and the top 20% of kinases are responsible for regulating 90% of those sites. More than 150 kinases have no assigned substrates. Consequently, mapping a set of regulated phosphosites onto a network of kinase-substrate interactions is naturally challenging. Some interesting new approaches are being used to address this question, in particular, CRISPR has been used to delete certain kinases in the genome, followed by phosphoproteomics to assess the effects on the phosphoproteome (Isobe et al., 2017; Isobe et al., 2020).

The studies reviewed here have used different methods to achieve visualization of PGE$_2$ signaling networks. In one article centered on PKA signaling (Giansanti et al., 2013), a PKA network was visualized in Cytoscape by using the proteins identified as potential PKA substrates in the study, and expanding with protein-protein interactions derived from STRING and kinase-substrate interactions from PhosphoSitePlus. Another article (de Graaf et al., 2014) used a similar approach to arrive at predicted networks and complexes of predicted PKA and CK2 substrates. In addition, the authors manually constructed a basic PGE$_2$ signaling network using kinases and substrates seen to be regulated in their study and connecting these using information from UniProt and PhosphoSitePlus as well as information about the temporal regulation of the kinases and substrates from their study (see

Table 2). Such manual curation is feasible for relatively limited networks but is unrealistic for mapping entire phosphoproteomics datasets onto a potential network.

In our recent paper, we used a different approach to predict possible signaling networks based on the sites seen to be regulated by the four different PGE$_2$ receptors (Lone et al., 2021). In this approach, PHONEMeS (Terfve et al., 2015) was applied by combining phosphoproteomic data with a network of directed protein-protein and kinase/phosphatase to substrate interactions representing prior knowledge. This resulted in network models for stimulation of one or multiple PGE$_2$ receptors simultaneously. This modeling approach provides a new way of constructing possible signaling networks for large phosphoproteomics datasets (Table 2). In this case, the modeled networks helped visualize the differences in signaling between the different EP receptors. From the modeled networks it also appeared that PGE$_2$ signaling in CD4 cells is relatively similar to that in CD8 cells, with the main difference being in the intensity of the signaling, not the overall layout of the signaling pathways. Naturally, we see evidence for the canonical G-protein dependent pathways, including PKA, PLC/PKC and PI3K/Akt, but another major prediction from these models was that a large part of the PGE$_2$ signaling was modeled as going through G-protein independent pathways, such as beta-arrestin.

Distinct PGE$_2$ responses across T cell subtypes

The PGE$_2$ phosphoproteomics studies in T cells were carried out either in the Jurkat T cell line (de Graaf et al., 2014; Giansanti et al., 2013), or in primary T cells (Lone et al., 2021; Oberprieler et al., 2010). Many features of PGE$_2$ signaling pathways, including kinase nodes and pathways involved, appear to be shared between the cell line and primary cells. The phosphosites regulated by PGE$_2$ also show significant overlap between studies in cell lines versus studies in primary cells (Figure 2), where the Giansanti et al., and De Graaf et al. studies were carried out in the Jurkat cell line and the Lone et al. and Oberprieler et al. studies were performed in primary T cells. We note that there are also a number of phosphosites specific to each study, likely due to specific experimental or technical conditions in each experiment. For instance, De Graaf et al. and Lone et al. were label-free, while Giansanti et al. and Oberprieler et al. used stable isotope dimethyl labeling for quantitation, which could introduce some differences in what peptides were detected. In addition, Giansanti et al. differed from the other studies in that it used a PKA motif antibody for enrichment, which would also naturally have a large impact on what peptides were detected. Given the differences in methodological approaches and cell types investigated, it is unsurprising that the overlap between the studies is relatively modest.

Interestingly, there also appear to be certain differences in the signaling between primary T cell subtypes when directly compared against each other, underlining the complexity of PGE_2 signaling in T cells. In particular, the intensity and to some extent duration of signaling appears to be the strongest in CD8+ cells, and in particular in CD8 memory cells (Lone et al., 2021; Oberprieler et al., 2010). One study found that similar signaling pathways appear to be present in the different T cell subsets, but that the intensity and also relative contributions of these pathways likely differs across cell types. For instance, CK2 appears to have a stronger contribution in CD4 than in CD8 cells (Lone et al., 2021), in line with a literature on possible roles of CK2 in CD4 cells (Gibson and Benveniste, 2018). Studies in cell types beyond T cells have shown that PGE_2 signaling in other cell types also shares certain features with the signaling observed in T cells. For instance, a PGE_2 phosphoproteomics study in fibroblasts showed that PKA was a key node in the signaling network here too, and that PGE_2 was involved in the regulation of many of the same cellular functions as in T cells, for instance cytoskeletal structures (migration/motility), regulators of G-protein coupled receptor function, protein kinases, and transcriptional/translational regulators (Gerarduzzi et al., 2014; Lone et al., 2021). PGE_2 is thought to also play an important role in regulating the functions of a number of immune cells involved in cancer (Wang and DuBois, 2013), though so far the mechanisms behind these roles have not been studied with phosphoproteomics methods.

Temporal patterns in PGE_2 signaling

Several of the phosphoproteomics studies of PGE_2 signaling in T cells included multiple timepoints (de Graaf et al., 2014; Giansanti et al., 2013; Oberprieler et al., 2010), allowing information also on temporal aspects of this process and from a modeling perspective introducing directional edges when mapping signal networks (Figure 3). In one study, 0-, 1- and 60-minute time points were used to study the temporal regulation of potential PKA substrates by PGE_2 (Giansanti et al., 2013). Five distinct temporal profiles were identified, of which three showed upregulation over time, one showed no regulation, and one profile showed downregulation at the 1- and/or 60-minute timepoints. This downregulation of PKA substrate phosphorylation in response to PGE_2 stimulation in a small subset of regulated sites is counterintuitive, but could be due to a post-activation phenomenon. In our recent study, a PKA-like motif was observed in many downregulated sites at the 10-minute timepoint, perhaps indicating a similar phenomenon at this time (Lone et al., 2021).

In another study, label-free quantitation allowed the monitoring of additional timepoints, namely 0, 5, 10, 20, 30 and 60 minutes (de Graaf et al., 2014). The authors note that including these additional timepoints may be useful in identifying transiently activated substrates and enabled the grouping of sites according to temporal regulation patterns, with five clusters showing different upregulation

patterns and three showing different downregulation patterns. Interestingly, different kinases appeared to be active at the different timepoints, as evidenced by distinct kinase predictions and regulated phosphorylation motifs in the different clusters and also introducing the possibility of signal amplification by serially activated kinases along the same pathway (Figure 3). For instance, basic motifs were mostly upregulated at the early timepoints such as 5 and 10 minutes, while acidic motifs were more enriched at 20 minutes and later and proline-directed motifs didn't show any particular temporal patterns. This is supported by kinase predictions, which indicate upregulation of basophilic kinases, for instance PKA and PKC, at early timepoints and acidophilic kinases, such as CK1 and CK2, at 20 minutes and later timepoints. In some cases, these temporal profiles could aid functional delineation and kinase function assignments (Figure 3). For instance, CLK1 and CLK4 displayed a distinct temporal profile in this study, with activation at an intermediate timepoint, and for this temporal cluster, the term mRNA processing was highly enriched in GO analysis, aligning well with what is known about CLK1 function in the mRNA spliceosomal complex. Interestingly, a greater number of phosphosites was regulated at later timepoints than at earlier timepoints in this study, suggesting amplification of the original signaling response and that kinases activated at later timepoints may thus constitute a broader, secondary response to PGE$_2$ (Figure 3). On the other hand, some kinases may become inactive over time, leading to lower phosphorylation levels of the final substrate, such as in the case of S3 on CFL, which showed decreased abundance at late timepoints, likely due to inactivation of the intermediate nodes RhoA, ROCK or LIMK.

A further study in primary T cells used the timepoints 0, 10 and 60 minutes, and saw similar temporal patterns as above (Oberprieler et al., 2010), with most phosphosites clustering into groups with maximum phosphorylation at 10 or 60 minutes. This study had another unusual feature in that it combined mass spectrometry-based phosphoproteomics with phosphoflow to get both the global view from phosphoproteomics as well as a more high-throughput and detailed view from phosphoflow, including the ability to get detailed temporal information. The six phosphosites examined by phosphoflow exhibited distinct kinetics. A general PKA substrate antibody showed maximum phosphorylation at 10 minutes and GSK3a pS21, a PKA substrate, had a similar temporal profile, agreeing with the results described above with early maximal activation for PKA (de Graaf et al., 2014; Giansanti et al., 2013). Of the other sites, some came up early (HSP27 pS78 at 3 minutes), intermediate (S6 ribosomal protein pS235/236) and others late (Histone H3 pS10). Notably, for the phosphosites also seen by mass spectrometry, the temporal patterns were similar between the two techniques, confirming the usefulness of this combination of techniques to get more detailed temporal information in a high-throughput manner.

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A more recent study of PGE$_2$ signaling also used the combined phosphoproteomics and phosphoflow cytometry approach, now in order to individually characterize PGE$_2$ signaling through each of its four receptors on T cells and how this differs between T cell subtypes (Lone et al., 2021). This study focused on a single, early/intermediate timepoint, namely 5 minutes, and observed both basic, acidic and proline-directed motifs at this timepoint. Kinases found in other studies to be primarily "early" or "late" were both predicted at this timepoint (de Graaf et al., 2014), indicating that at this intermediate timepoint it may be possible to observe both the tail end of early signaling as well as the beginning of late-onset signaling events. The phosphoflow cytometry portion of this study confirmed a distribution of temporal profiles in PGE$_2$-regulated phosphorylation sites, with some phosphosites showing maximum regulation at early timepoints (e.g. Vimentin pS38, VASP pS157), intermediate timepoints (e.g. S6RP pS240, NDRG1 pT346) or late timepoints (e.g. CREB pS133, Histone H3 pS10, pS28). Interestingly, some of the proteins investigated had multiple phosphosites and these were seen to be regulated with different dynamics, for instance in the case of S240 and S235/236 on S6RP. Of the potential PKA substrates, a majority were early, including GSK3a pS21, VASP pS157, Vimentin pS38 and HSP27 pS78, with a few showing later activation, including Histone H3 pS10 and CREB pS133. The differing kinetics could be due to cellular localization or contributions from kinases beyond PKA that have different activation dynamics. This study was also able to highlight some of the difference between receptors and cell types when it comes to the dynamic signaling response to PGE$_2$. In particular, the EP$_2$ receptor signaled with stronger and more prolonged dynamics than the EP$_4$ receptor, which we hypothesize is due to a weaker functional coupling to cAMP and PKA as well as less rapid receptor internalization. In addition, certain T cell subtypes, in particular CD8CD45RO, appeared to have stronger and more prolonged signaling responses than other cell subtypes.

Functional output of PGE$_2$ signaling in T cells

One of the major current challenges in phosphoproteomics is translating information on regulated phosphosites into effects on cellular function (Needham et al., 2019). While more than 200,000 phosphosites are currently known (Hornbeck et al., 2012), fewer than 3% of identified human phosphosites have a reported function (Needham et al., 2019). Several approaches can be used to identify or predict the functionality of particular phosphorylation sites, with one recent approach using machine learning to predict which phosphosites are likely to be functional (Ochoa et al., 2019). In the PGE$_2$ signaling studies in T cells, the function of individual phosphosites has largely been explored using either predictive software such as Predict Functional Phosphosites (PFP) (Xiao et al., 2016) or using GO analysis (Ashburner et al., 2000; Bindea et al., 2009; Carbon et al., 2019), where

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function prediction is at the protein level. If a site is thought to be functionally important through bioinformatic analysis, siRNA knockdown or CRISPR gene editing can be used to delete the phosphosite or replace it with a non-phosphorylatable amino acid or a phosphomimetic to further elucidate the functional role of the phosphosite in cells (Aggarwal et al., 2019; Dukic et al., 2018; Liu et al., 2020).

Through the four phosphoproteomics studies of PGE_2 signaling in T cells, a number of functional outputs of this signaling have been predicted or confirmed. In particular, the intersection of PGE_2 signaling with TCR signaling has been a theme in several of the studies. In one study, many proteins involved in TCR signaling, such as CARD11, PLCG1, WIPF1, GRAP2, NFATC2, FYB1 and NCK1, were found to be phosphorylated in response to PGE_2 (Oberprieler et al., 2010). In our more recent study, we observed that all four EP receptors regulate the phosphorylation of proteins contained in the GO term "TCR signaling pathway" and that stimulation with PGE_2 or a specific agonist of the EP_2 receptor gives enrichment of this GO term. TCR signaling proteins whose phosphorylation state was regulated by PGE_2 or EP_2 agonists include: ARHGEF7, CARD11, CD247, FYB1, FYN, GRAP2, INPP5D, LAT, LCK, LCP2, LIME1, MAP3K7, NCK1, NCOR2, PAG1, PAK2, PDPK1, PIK3R1, PLCG1, PRKCQ, PSMA5, PSMD11, PSMD2, PSMD3, PTPN22, PTPRC, RFTN1, TESPA1 and WAS. A few proteins, namely GRAP2, PAG1, PLCG1 and PSMD3, were unique to PGE_2, while all other proteins regulated by PGE_2 were also found in the EP_2 agonist regulated protein set, suggesting that much of the PGE_2 signaling that intersects with TCR signaling goes via the EP_2 receptor. Interestingly, in this study we observed regulation of the inhibitory Lck pY505 site only in CD4 cells, where it was elevated in all conditions. This site has been shown to partake in an inhibitory PGE_2 pathway that intersects TCR signaling. This pathway is triggered by EP_2 or EP_4, and proceeds via cAMP-mediated PKA activation, which leads to Csk phosphorylation and inhibitory phosphorylation of Lck at Y505 (Ruppelt et al., 2007; Vang et al., 2001; Wehbi and Tasken, 2016).

A number of other functions were also predicted by GO analysis in our study, including cytoskeleton organization, mRNA processing, cell-cell adhesion, cell polarity and small GTPase-mediated signal transduction, which were enriched in all stimulation conditions in CD8 cells. Some of these functions appear to be conserved across cell types, as similar functions were also predicted in fibroblasts (Gerarduzzi et al., 2014). In terms of more specific immune functions, GO analysis showed enrichment for T cell activation (upon EP_1, EP_3 and PGE_2 stimulation), establishment of T cell polarity (EP_3, EP_4, PGE_2), thymic T cell selection/T cell differentiation in thymus (EP_1, EP_3, EP_4), lymphocyte migration (EP_4) and lymphocyte proliferation (EP_3), which is in line with some of the known functions of PGE_2 in T cells (Lone and Tasken, 2013).

In addition, one study (Lone et al., 2021) used the "Predict Functional Phosphosites" (PFP) algorithm to predict which regulated phosphosites were likely functional. While there is some discussion about whether all phosphosites are in fact functional or whether some simply result from off-target effects of kinases (Lienhard, 2008), the PFP algorithm aims to identify the phosphosites most likely to have biological functions based on conservation, kinase association and structure information. In this study, PFP thus yielded a list of potentially biologically active phosphosites, as well as a number of possible functional outputs of PGE_2 signaling for those sites already annotated with biological function. Conveniently, the study also provides information on cell types in which these phosphorylation events occur and the receptor stimulation conditions under which they are most likely to be regulated. This provides a very useful starting point for functional studies in T cells.

Manual assessment of the functional roles of individual sites by consulting the literature can also be an option if only looking at a limited number of sites, and importantly provides crucial information compared to STRING or GO analysis, which do not have directionality. This is a limitation in terms of pathway and network modeling. To identify sites of particular importance, one strategy might be to consider the magnitude of the change in phosphorylation level in response to the stimulus, and focus on changes that have a larger fold change or are more statistically significant. Another, strategy might be to include temporal information to get directional information on edges (Figure 3). However, manual curation is required in this process, as fold changes are influenced by many factors, including kinetics of phosphorylation, position in signaling pathway, cellular location and turnover, and therefore fold change does not always correlate with more meaningful or functionally significant phosphorylation events. The PhosphoSitePlus database (Hornbeck et al., 2012) provides a reference for what is currently known about the function of specific phosphorylation events. Most of the sites monitored by phosphoflow in the two combined phosphoproteomics/phosphoflow cytometry studies are relatively well characterized in the literature and are also annotated in PhosphoSitePlus and many of them have known biological functions, for instance in cytoskeletal function, T cell polarization, transcription and translation, which aligns well with the GO analysis in the latter study (Lone et al., 2021). Another study (de Graaf et al., 2014) also found many of the same functions to be regulated by PGE_2. Here, manual inspection of phosphosites revealed some of the functional associations, such as S2152 on FLNA and S16 on STMN1 implying the intersection of PGE_2 signaling with cytoskeleton reorganization and upregulation of pS118 in BAD implying downregulation of apoptosis. Also, gene ontology analysis was employed and showed the regulation of endocytosis, RNA processing and DNA-related terms. Interestingly, these terms were upregulated in different temporal clusters of phosphosites, indicating regulation of different processes at different timepoints after PGE_2 stimulation, likely correlating with activation of different kinases at different timepoints.

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Using GO-based functional annotation of substrate interaction networks for the kinases CK2 and PKA, the paper found that the CK2-specific substrate interaction networks DNA repair and mRNA translation occurred only in the temporal clusters with delayed upregulation, matching CK2's observed late upregulation. Similarly, the PKA-specific substrate interaction network "T cell signaling" was found only in the temporal cluster of phosphosites that remained upregulated over time. Interestingly, another study found that PGE_2-triggered PKA substrates were involved in a number of other biological processes as well, including transcription, translation, cytoskeletal function, kinase and phosphatase function and more (Giansanti et al., 2013), illustrating how one kinase node in a signaling network can regulate many different biological processes, and underlining the key role of the PKA node in PGE_2 functional regulation.

The known functions of PGE_2 in T cells, including in differentiation, proliferation, apoptosis and more (Lone and Tasken, 2013), align well with the biological functions identified in these proteomics studies, for instance cell cycle regulation, cytoskeletal remodeling, transcription and translation and in particular the immunological functions identified, such as T cell activation, establishment of T cell polarity, thymic T cell selection/T cell differentiation in thymus, lymphocyte migration and lymphocyte proliferation.

Together, these functional interpretations have led to the strong implication of PGE_2 signaling in certain important cellular and immunological processes, such as regulation of RNA- and DNA-related processes, cytoskeleton remodeling, T cell signaling and more. Importantly, these studies have generated specific hypotheses that can then be tested based on evidence of PGE_2 involvement in specific processes. Furthermore, some of the studies have also provided more detailed information about the mechanisms and kinetics with which PGE_2 regulates this diverse biology, for instance through which receptors and kinases the functions may be regulated and with what temporal dynamics. One can envision that more directed functional studies can now be carried out on specific identified regulated phosphosites that, through these phosphoproteomics studies, have been strongly implicated in the regulation of specific processes by PGE_2.

Perspectives and Future Directions

The phosphoproteomics studies described here have significantly contributed towards an improved understanding of PGE_2 signaling in T cells. This includes insights into specific pathways triggered by the four PGE_2 receptors, which kinase nodes are active in these pathways and how receptors and pathways crosstalk to form signaling networks upon PGE_2 stimulation. Thanks to temporal phosphoproteomics studies and complementary phosphoflow cytometry studies, we also have

detailed information about the temporal regulation of many of the signaling pathways, as well as about differences in signaling between different T cell subtypes. Further, the studies have suggested possible functional outcomes of PGE_2 stimulation in T cells. Together, they constitute a solid platform for targeted studies of specific PGE_2-triggered pathways in T cells and enable more directed functional studies for particular PGE_2-triggered pathways. These PGE_2 signaling studies further showcase some of the common current challenges and future directions of phosphoproteomics studies. In particular, they illustrate that the nature of the signaling response from a single given stimulus, in this case PGE_2, is not that of a single, linear pathway, but rather a complex, interconnected network which integrates multiple signals, in this case from multiple receptors and multiple kinase nodes (Needham et al., 2019). In this way, phosphoproteomics becomes a key tool in understanding the behavior of entire systems rather than individual, isolated pathways and the temporal and quantitative nature of the technique is an important asset in understanding how these networks are regulated.

The studies also illustrate some of the current challenges in phosphoproteomics, including those in understanding the upstream regulation and downstream function of phosphosites (Needham et al., 2019). Progress in mapping more kinase-substrate interactions (Sugiyama et al., 2019), as well as in the development of software and modeling methods that allow the organization of phosphoproteomics data into predicted pathways and networks (Kotecha et al., 2010; Kramer et al., 2014; Raaijmakers et al., 2015; Terfve et al., 2015) will continue to contribute towards the successful interpretation of phosphoproteomics studies. As for the issue of predicting function based on specific regulated phosphosites, methods for predicting which phosphosites are functional are constantly improving (Beltrao et al., 2012; Ochoa et al., 2019; Xiao et al., 2016) and lists of phosphosites with known function are also continuously lengthening (Hornbeck et al., 2012). These advances will keep improving the translation of phosphoproteomics data into actionable functional predictions. There are also some challenges in phosphoproteomics specific to immune cells. For instance, depending on the particular cell type, the number of cells obtained can be quite low. For instance, Tregs may need to be expanded prior to analysis due to their relatively low abundance, which could alter the signaling properties of the cells (Lone et al., 2021). As the sensitivity of mass spectrometers keeps increasing, however, this will become less of a hurdle (Alvarez-Salamero et al., 2017; Needham et al., 2019; Riley and Coon, 2016).

We envision that future directions in phosphoproteomics studies of PGE_2 signaling in T cells may include further investigations of signaling differences between T cell subtypes. For instance, the advent of CyTOF is facilitating the simultaneous observation of multiple phosphorylation events in ever-smaller subsets of cells (Gullaksen et al., 2019; Helou and Salomon, 2015), even within a single

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cell, thus facilitating a better understanding of heterogeneity in signaling responses between cells and between cell types. Single-cell mass spectrometry is also under development and may eventually include the possibility of looking for phosphorylation events in single cells (Marx, 2019; Specht et al., 2019). Similarly, as the sensitivity of mass spectrometry approaches keeps increasing and less material is required for analysis, it will also be possible to study signaling in smaller and smaller T cell subsets without necessitating cell expansion prior to analysis (Alvarez-Salamero et al., 2017). Interesting subsets to analyze for signaling differences might be subsets of helper and cytotoxic T cells such as Tregs, Th17 and others, to be able to correlate the differing functional effects of PGE_2 in these cell types, for instance in T cell differentiation. It would also be interesting to study any changes in PGE_2 signaling in contexts with prolonged dysregulation of PGE_2, such as certain cancers and chronic inflammation, for instance in cells that express exhaustion markers or have upregulated immune checkpoints. It would also be interesting to more comprehensively study how PGE_2 signaling differs in cancer patients with upregulated versus normal PGE_2 levels (Moltu et al., 2017). In this and possibly other contexts, it would be a valuable extension from the current studies to also perform integrated phosphoproteomics/transcriptomics/proteomics studies, with the aim of correlating changes in phosphorylation patterns with later, possibly permanent changes in gene and protein expression levels. A few examples of such integrated studies on other topics are now available (Gao et al., 2019; Rotival et al., 2015; Zadora et al., 2019).

Another direction for PGE_2 signaling studies might be the comparison with other cell types beyond T cells to assess commonalities and differences in signaling and in functional outcome of the signaling in these cell types. While PGE_2 signaling by phosphoproteomics has been mostly studied in T cells to date, one phosphoproteomics study in fibroblasts is available (Gerarduzzi et al., 2014) and many of the findings in this study echo the results in T cells. In particular, PKA has a key role in PGE_2 signaling networks in this cell type as well, and PGE_2 regulates functional processes such as cytoskeletal rearrangement, GPCR function, kinases and transcription/translation, similar to what was found in T cells. At the same time, PGE_2 signaling in fibroblasts has a net functional output that is naturally distinct from that in T cells, namely as an antifibrotic mediator, and this phosphoproteomic study gives new insight into molecular mechanisms for PGE_2 regulation of fibroblast activation and potential starting points for more targeted studies to elucidate these mechanisms. Interestingly, PGE_2 is known to have important and distinct functional roles in many other cell types, for instance NK cells (Holt et al., 2011) and B cells (Murn et al., 2008) and it may be fruitful to use phosphoproteomic approaches to gain further insight into the molecular regulation of these functions. In addition, gene expression profiles for the different EP receptors indicates that their relative expression levels vary significantly between tissues (Consortium, 2013), so it would also be

interesting to explore PGE_2 signaling by phosphoproteomics in other tissue types with differing relative receptor distribution patterns. Expanding the perspective, it would also be interesting to use phosphoproteomics to understand the signaling of other GPCR families that form signaling networks, for instance the adrenergic receptor family, which also contains two $G\alpha_s$, one $G\alpha_i$ and one $G\alpha_q$ receptor(Fujino and Regan, 2006; Hall, 2004). Exploring signaling in this or other GPCR signaling networks through phosphoproteomics would be a worthwhile community project.

Already, the results from the phosphoproteomics studies have been used as a basis for more targeted biochemical and functional studies (Burdyga et al., 2018; Moiseeva et al., 2019; Moltu et al., 2017) and this is also a possible direction for further research. The most recent phosphoproteomics study of PGE_2 signaling in T cells assessed the relative contributions of the four different EP receptors to PGE_2 signaling in subtypes of T cells, as well as the overlap of these and the relative contributions of G-protein -dependent and -independent signaling. It would be interesting to further characterize possible crosstalk and synergies between these receptors in molecular detail, for instance using phosphoflow cytometry, and such studies would be aided by the ever-increasing repertoire of agonists and antagonists of the different receptors (Markovic et al., 2017; Woodward et al., 2011). Through such molecular studies one might also be able assess the effect of different kinase inhibitors and/or disruptors of known PGE_2 signaling pathways (Stokka et al., 2009; Torheim et al., 2009) to see how this affects specific pathways as well as PGE_2 signaling more generally. Another direct continuation of these studies would be to test the predicted functional outcomes of PGE_2 signaling in more detail, for instance by using lists of PGE_2-regulated sites with known biological function obtained from the phosphoproteomics studies and characterizing the mechanisms and pathways by which PGE_2 might regulate function through these sites. GO analyses from these phosphoproteomics studies could also be used as a basis for studies of the mechanisms behind how PGE_2 regulates specific T cell biological functions. While many of the current studies have focused on PKA as a major node in PGE_2 signaling, it would also be interesting targeting other kinases identified as important, for instance through targeted phosphoproteomics studies.

All in all, the phosphoproteomics studies carried out in the previous decade have provided a trove of information and insights into PGE_2 signaling pathways in T cells and we hope they will continue to spark new studies that further elucidate how PGE_2 signaling pathways and networks behave and may be targeted under normal and disease conditions.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Lone, A.M. and Taskén, K.

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Legends to Figures and Tables

Table 1. Current clinical trials with EP$_4$ antagonists. Overview of current clinical trials with EP$_4$ antagonists in cancer. To identify clinical trials, a search was performed on clinicaltrials.gov with the keyword "Prostaglandin E2", selecting for interventional studies and condition=cancer/neoplasm/tumor. In addition, searches were also performed with the names of all EP$_4$ antagonists listed in the following database: https://www.guidetopharmacology.org, as well as EP$_4$ antagonists found by searching the NCI Drug Dictionary: https://www.cancer.gov/publications/dictionaries/cancer-drug.

Table 2 List of phosphoproteomics studies of PGE$_2$ signaling. The table gives an overview of the studies discussed in this review article. In addition to the four phosphoproteomics studies of PGE$_2$ signaling in T cell, two related articles have also been included.

Table 3. Proteins from GO Term T cell receptor signaling pathway whose phosphorylation is regulated by PGE$_2$. Table shows proteins contained in GO Term T cell receptor signaling pathway that have one or more phosphosites that are regulated by PGE$_2$ or one of the EP receptor agonists in one or more of the phosphoproteomics studies reviewed here. Commonly used alternative names for the proteins shown in parenthesis.

Figure 1. PGE$_2$ signaling regulates phosphorylation of proteins involved in TCR signaling. Phosphosites regulated by PGE$_2$ in the four different phosphoproteomics studies were queried against proteins included in the GO Term "T cell receptor signaling pathway" (grey). TCR signaling proteins (green) have phosphosites regulated by PGE$_2$ or one of the EP receptor agonists in one or more of the phosphoproteomics studies. Asterisks indicate proteins not included in GO Term TCR signaling (GRAP2=GADS, NCK1), but known to be involved in TCR signaling and regulated in Oberprieler et al.

Figure 2. Overlap in phosphorylation sites regulated in the four studies. Venn diagrams showing the overlap between the four phosphoproteomics studies of PGE$_2$ signaling in T cells. For the Lone et al. study, all regulated sites, also from stimulation with individual EP agonists, were included. As evident from this illustration, some of the regulated sites are specific to each study, and some are shared between studies. Left panel does not include de Graaf et al., which was a targeted study of PKA substrates. Right panel includes all four studies described here.

Figure 3. Schematic showing kinetics of phosphorylation for phosphosites at different relative positions in a phosphorylation cascade. Phosphorylation time courses may in some cases be used to infer the position of a phosphosite in a signaling pathway, with early phosphorylation suggesting a position near the top of a signaling cascade (red) and later phosphorylation suggesting a position further down in the cascade (blue).

Table 1. Current clinical trials with EP4 antagonists.

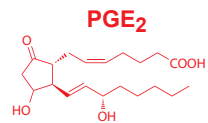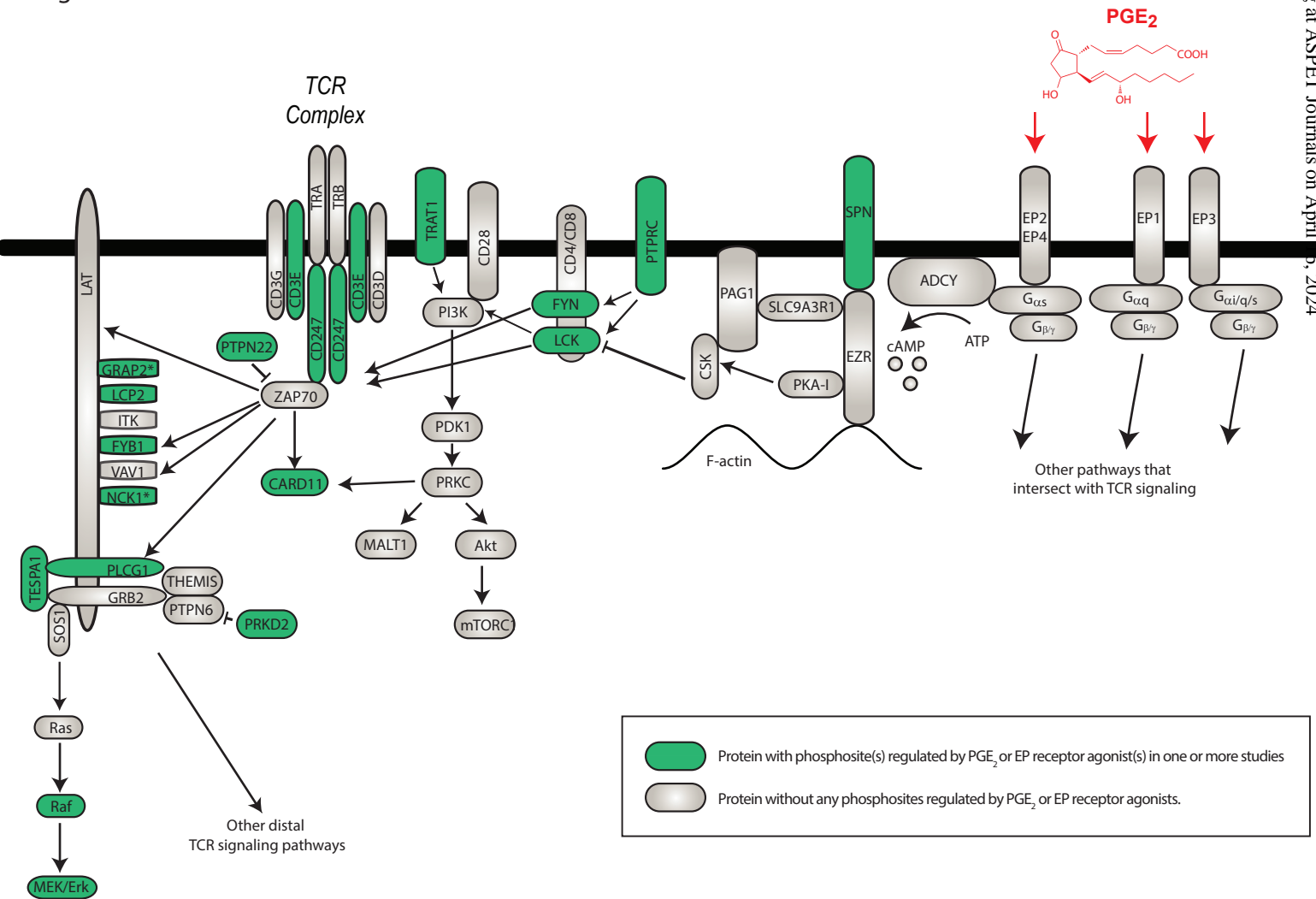| Trial number	Title	Conditions	EP4 Antagonist	Combination with	Status	Phase
NCT04344795	Phase 1a/1b Study of TPST-1495 Alone and With Pembrolizumab in Subjects With Solid Tumors	Solid Tumor, Microsatellite-stable Colorectal Cancer (MSS CRC), Adenocarcinoma of the Lung, Squamous Cell Carcinoma of Head and Neck, Bladder Cancer, Triple Negative Breast Cancer, Gastric Cancer	TPST-1495	Pembrolizumab	Recruiting	1
NCT03658772	Grapiprant and Pembrolizumab in Patients With Advanced or Progressive MSS Colorectal Cancer	Microsatellite Stable Colorectal Cancer	Grapiprant	Pembrolizumab	Recruiting	1
NCT03696212	Grapiprant (ARY-007) and Pembrolizumab in Patients With Advanced or Metastatic Post-PD-1/L1 NSCLC Adenocarcinoma	Non-small Cell Lung Cancer Adenocarcinoma	grapiprant and	pembrolizumab	Recruiting	1, 2
NCT02538432	Phase II Trial of EP4 Receptor Antagonist, AAT-007 (RQ-07; CJ-023,423) in Advanced Solid Tumors	Prostate Cancer Non-Small Cell Lung Cancer Breast Cancer	RQ-00000007	Gemcitabine	Withdrawn	2
NCT03163966	A Study of the EP4 Antagonist CR6086 in Combination with Methotrexate, in DMARD-naïve Patients With Early Rheumatoid Arthritis	Rheumatoid Arthritis, DMARD-naive and Early Disease Patients	CR6086	Methotrexate	Unknown	**2**
NCT00957983	EP4-receptor Antagonism and Prostaglandin E2 (PGE2) in a Human Headache Model	Headache, Migraine	BGC20-1531		Completed	**1,2**

Table 2 List of phosphoproteomics studies of PGE$_2$ signaling.

Study	Cell type	Labeling	Enrichment	Data and Pathway Analysis Approaches	Notes
Oberprieler et al., 2010. *High-resolution Mapping of Prostaglandin E2–dependent Signaling Networks Identifies a Constitutively Active PKA Signaling Node in CD8+CD45RO+ T Cells.* PMID 20558615	Primary CD3+	Stable isotope dimethyl labeling		PhosphoSitePlus, NetworKIN, Scansite, Phosida, IceLogo	Combined w/phosphoflow.
Giansanti et al., 2013. *Interrogating cAMP-dependent Kinase Signaling in Jurkat T Cells via a Protein Kinase A Targeted Immune-precipitation Phosphoproteomics Approach.* PMID: 23882029	Jurkat	Stable isotope dimethyl labeling	PKA motif antibody enrichment	Ingenuity Pathway Analysis, Cytoscape, STRING, Phosphositeplus	
De Graaf et al., 2014. *Single-step Enrichment by Ti4+-IMAC and Label-free Quantitation Enables In-depth Monitoring of Phosphorylation Dynamics with High Reproducibility and Temporal Resolution.* PMID 24850871	Jurkat	Label-free		STRING, NetworKIN, IceLogo, Gene Ontology analysis	
Lone et al., 2021. *Systems Approach Reveals Distinct, Joint Signaling Networks of the Four PGE$_2$ Receptors in T Cells*	Primary CD4+, CD8+, Tregs	Label-free		NetworKIN, IceLogo, Phonemes, STRING, PhosphositePlus, Predict Functional Phosphosites	Combined with phosphoflow. Individual stimulation of 4 PGE$_2$ receptors.
Gerarduzzi et al., 2014. *Quantitative Phosphoproteomic Analysis of Signaling Downstream of the Prostaglandin E2/G-Protein Coupled Receptor in Human Synovial Fibroblasts: Potential Antifibrotic Networks.* PMID: 25223752	Primary synovial fibroblasts	Label-free	PKA motif antibody enrichment	Ingenuity Pathway Analysis	Related study.
Beltejar et al., 2017. *Analyses of PDE-regulated phosphoproteomes reveal unique and specific cAMP-signaling modules in T cells.* PMID: 28634298	Jurkat	Label-free		NetPhorest, Predict Functional Phosphosites, STRING, Gene Ontology Analysis	Related study. Combined stimulation with PDE inhibitors and PGE$_2$

Table 3. Proteins from GO Term T cell receptor signaling pathway whose phosphorylation is regulated by PGE₂.

TCR Signaling Protein	Oberprieler	Giansanti	de Graaf	Lone	Conditions Regulated in Lone
BRAF		✓			
CARD11 (Carma1)	✓		✓	✓	CD8 EP1, EP2, EP3, EP4, PGE2
CD247 (CD3ζ)			✓	✓	CD8 EP1, EP2, EP3
CD3E			✓		
ELF1			✓		
FYB1 (ADAP)	✓		✓	✓	CD8 EP2, EP4
FYN				✓	CD8 EP2
GATA3			✓		
LCK	✓		✓	✓	CD8 EP2 / CD4 EP1, EP2, EP3, EP4, PGE2
LCP2 (SLP76)	✓			✓	CD8 EP2
LIME1				✓	CD8 EP1, EP2, EP3, EP4, PGE2
MAPK1			✓		
PLCG1	✓			✓	CD8 PGE2
PRKD2	✓		✓	✓	CD4 EP4
PTPN22 (LYP)			✓	✓	CD8 EP2
PTPRC (CD45)			✓	✓	CD8 EP2
RFTN1				✓	CD8 EP2
RNF31			✓		
SPN (CD43)	✓		✓	✓	CD8 EP1, EP2, EP3, EP4, PGE2 / CD4 EP1, EP2, EP3, EP4, PGE2
TESPA1				✓	CD8 EP2
TRAT1 (TRIM)			✓		
WNK1	✓	✓		✓	CD8 EP2, EP4

Figure 1

TCR Complex

PGE₂

Legend:
- Protein with phosphosite(s) regulated by PGE₂ or EP receptor agonist(s) in one or more studies
- Protein without any phosphosites regulated by PGE₂ or EP receptor agonists.

Figure 2

Figure 3